The Toxin–Antidote Model of Cytoplasmic Incompatibility: Genetics and Evolutionary Implications

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Abstract

Wolbachia bacteria inhabit the cells of about half of all arthropod species, an unparalleled success stemming in large part from selfish invasive strategies. Cytoplasmic incompatibility (CI), whereby the symbiont makes itself essential to embryo viability, is the most common of these and constitutes a promising weapon against vector-borne diseases. After decades of theoretical and experimental struggle, major recent advances have been made toward a molecular understanding of this phenomenon. As pieces of the puzzle come together, from yeast and *Drosophila* fly transgenesis to CI diversity patterns in natural mosquito populations, it becomes clearer than ever that the CI induction and rescue stem from a toxin-antidote system. Further, the tight association of the CI genes with prophages provide clues to the possible evolutionary origin of this phenomenon and the levels of selection at play.

Every living organism is a chimera of different evolutionary lineages living in more or less tight association. Arthropods are emblematic of that rule and often carry bacteria within their own cells, transmitted from mothers to offspring with the egg cytoplasm. These may offer benefits, such as vital nutrients to hosts feeding exclusively on sap or blood [1,2], and thus become essential parts of a new whole, but also often colonize host populations through selfish strategies, maximizing their own fitness regardless of possible detrimental effects to hosts [3,4]. Cytoplasmic incompatibility (CI, Figure 1, box 1) is one such strategy, which has likely contributed in large part to the radiative success of *Wolbachia* bacteria, now present in about half of all arthropod species [5–7].

CI genetics

While *Wolbachia* and CI were both discovered a long time ago in *Culex pipiens* mosquitoes [8–11], the causal link between the two was only made decades later [12,13]. By that time, early models had clarified the invasion dynamics of CI [14], that were later extended [15] and calibrated with empirical data [16,17], but only in the 1990s was a formal mechanistic model proposed [18–20]. The fact that sperm from *Wolbachia*-carrying males kills uninfected but not infected embryos upon fertilization is compatible with a toxin-antidote model (hereafter TA model) (Figure 2, Key Figure). The toxin factor, deposited in maturing sperm, would kill the embryos unless they are rescued by the antidote. Although concurrent explanations were also proposed [21,22], the discovery of *Wolbachia* strains capable of rescuing CI without inducing it further supported the notion that this phenomenon should involve two distinct factors [23,24]. The observations of independent effects of distinct *Wolbachia* strains, either in the context of multiple infections or mutual incompatibility between different *Wolbachia* strains,

further suggested the toxin and antidote should interact specifically, in a lock-and-key manner [25]. This framework generated a set of testable predictions that fueled the experimental quest to identify the CI genes, which was recently achieved [26–30].

The first evidence pointing to the two genes later established as genuine CI factors came from a sperm proteomic study based on the rationale that the hypothetical CI toxin should be present in infected males' mature sperm, even though the bacterium itself is not [26]. Inspired by earlier proteomic analyses of *Drosophila* sperm [31,32], this approach finally pinpointed the candidate CI genes in the mosquito *Culex pipiens*, where the high penetrance of CI associated with the *w*Pip *Wolbachia* strain predicted a toxin protein detectable by mass spectrometry. Here serendipity also played an essential role: the first CI protein identified in sperm in this study was later revealed not as the toxin gene product, as predicted, but as the antidote, the presence of which was not expected under the most parsimonious CI model [25]. Nevertheless, its synteny and co-transcription with another *Wolbachia* gene, later revealed as the CI toxin, was noted, as was the similarity of this locus with typical toxin-antidote systems, usually composed of two genes, the first of which, the antidote, is expressed at higher levels [33]. Proximity of these putative CI genes to prophages in the *w*Pip genome was also pointed out at that time [26].

The next major steps toward the demonstration that these genes and their homologs in *Drosophila* are responsible for the induction and rescue of CI came from a combination of approaches and model systems [27,28]. In line with the TA model, biochemical analysis and transgenic expression of the putative *w*Pip CI genes in yeast revealed that they encode a toxic deubiquitylase (DUB) and an inhibitor of this toxicity (DUBs are enzymes that specifically remove ubiquitin from ubiquitin-modified proteins). When transgenically expressed in uninfected *Drosophila* males crossed with uninfected females, these factors recapitulate CI

induction during the first embryonic mitosis [27]. Importantly, the two proteins were also found to bind tightly to one another in a cognate-specific manner [27] consistent with prior lock-and-key predictions [25]. At the same time, independent experiments involving the homologs of these genes from the *w*Mel *Wolbachia* strain, naturally infecting *D. melanogaster*, confirmed and complemented these results: their dual expression in uninfected *Drosophila* males induces a CI-like phenotype that, most importantly, is rescued by the presence of *w*Mel bacteria in females [28].

Before discussing the many questions raised by these results, a brief note is needed to avoid ambiguities stemming from different CI gene nomenclatures co-existing in the literature [27,28] (Figure 3). One proposal is that the operon inducing a CI-like phenotype when expressed in *Drosophila* should be called "*cid*", for "CI-inducing DUB" [27]. This function-based name was chosen to explicitly distinguish *cid* from *cin*, short for "CI-inducing nuclease", a paralogous operon in the *w*Pip genome, encoding a nuclease and showing a similar TA-like behavior in yeast, as well as polycistronic transcription [26,27]. Within each operon, the first gene, encoding the putative antidote, is labeled A (e.g., *cidA* or *cinA*) and the second, encoding the putative toxin, is labeled B. A contrasting nomenclature proposes that all the different CI-associated genes should be more neutrally noted as "CI factors" (*cif*), and that the different paralogs should be distinguished on the basis of phylogeny [28,34]. The "operon" designation is also questioned by these authors, primarily because the two transcripts can be measured at different levels, which could be indicative of different promoters [34]. Yet, no such distinct promoters have been identified and different expression levels of the two genes are not uncommon within bacterial operons [35]. In our view, the "operon" designation is thus appropriate in the present case. Regardless of this semantic debate, the two adjacent genes within each potential CI locus are labeled A and B in both nomenclature proposals.

Thus, *cidA* and *cidB* are synonymous with *cifA* and *cifB* (clade 1), respectively, and these constitute the major CI genes identified to date, recapitulating the phenotype in *Drosophila*. We see pros and cons in both nomenclatures and suggest they should be merged into a single system to avoid further confusion. The "*cif*" term (for CI factor) seems appropriate to designate CI genes in general and we will use it in that sense. To denote their different functional categories and evolutionary histories, we will distinguish *cid* (the **D**UB operon), from *cin* (the **n**uclease operon) and use this dichotomy to designate specific *cif* genes from any *Wolbachia* strain. Some operons have been predicted to carry both functions; we suggest these should be called "*cnd*" (Figure 3) [27,36]. When necessary, we will append the *Wolbachia* strain name as a superscript. In our opinion, this system merges positives of both previously proposed nomenclatures and fairly acknowledges the concomitant discovery of these gene pairs in two separate studies [27,28].

Following publication of the first two conclusive reports on *cif* genes, two major unsettled points remained [28,34]. First, the antidote activity of the CidA protein, although demonstrated for both CidA^{wPip} and CinA^{wPip} in yeast, was not established in *Drosophila*. A more recent report has now clarified this point: if expressed in sufficient amount during oogenesis, CidA^{wMel} was found to restore the viability of uninfected embryos fertilized by *w*Mel-carrying *Drosophila* males [29]. A second major question, still not fully settled as of this writing, is whether CidB can act alone as the CI toxin or needs some interaction with CidA to express toxicity. Transgenic expression in yeast indicates that the DUB activity of CidB^{wPip} has in itself a toxic effect, which is inhibited by co-expression of CidA^{wPip}. However, *Drosophila* lines expressing CidB^{wPip} alone were never obtained, making it impossible to directly assess its effect in this context [27]. A simple hypothesis, compatible with the idea that CidB alone is indeed the CI toxin, is that CidB^{wPip} can exert deleterious effects outside of the first embryonic

mitosis, where CI is usually first realized, and therefore requires co-expression of CidA, the antidote, in any tissue where CidB is present, and at sufficiently high dosage. Such a model would also help explain the paradoxical observation that first turned the spotlight toward the CI operon: the high dosage of CidA protein in mature sperm [26]. If this interpretation is correct, CI would result from removal or inactivation of the paternal CidA protein just before, or just after, fertilization.

At first sight, experiments involving transgenic expression of the *cid*$^{w\text{Mel}}$ genes in *Drosophila* argue against a toxic effect of CidB$^{w\text{Mel}}$ alone: lines expressing this protein were viable and the CI phenotype was recapitulated only by males expressing both CidB$^{w\text{Mel}}$ and CidA$^{w\text{Mel}}$ [28]. This result has been considered by some authors as arguing for a "two-by-one" model, in which CI induction would somehow rely on both CidA and CidB proteins, while rescue would rely on CidA only [29]. However, differences in the toxicity of CidB$^{w\text{Mel}}$ and CidB$^{w\text{Pip}}$ may reconcile these experimental results with a simple TA model. Although the *w*Mel *Wolbachia* strain may induce very high CI in a permissive host background (e.g., upon artificial transfer into *Drosophila simulans*) [37], it is well established that it has a low penetrance in its natural host [38], possibly as a result of past coevolution [39]. A low toxicity of CidB$^{w\text{Mel}}$ in *D. melanogaster* would explain why flies expressing this protein alone can survive. But why is CI not expressed in such transgenic lines? One possibility is that CidB$^{w\text{Mel}}$ is toxic enough to kill those maturing sperm cells where it is most highly expressed. It would follow that the surviving mature sperm would be precisely those where CidB$^{w\text{Mel}}$ is not sufficiently expressed to induce either sperm or embryonic death. This model could be tested by assessing if *D. melanogaster* males expressing CidB$^{w\text{Mel}}$ alone show a reduced sperm production.

Notably, the hypothesis that *cidB* may be deleterious in various cell types and developmental stages does not imply that the *Wolbachia* themselves express this gene in

many tissues of their native hosts. In other words, the pattern of expression of *cidB* in transgenic insects says nothing about its natural expression in *Wolbachia*-infected hosts. The fact that viable uninfected offspring can often be obtained through imperfect *Wolbachia* transmission or antibiotic curing actually suggests that in a natural context, the CI toxin is not expressed in all tissues. At present, one cannot exclude a "two-by-one" model, where both CidA and CidB proteins would be required to produce a toxic effect, but this would imply drastically different effects of CidA in sperm (where it would contribute to toxicity) and in the eggs (where it is known to act as an antidote). In our view, and as discussed above, a model where CidB and CidA act respectively as toxin and antidote is more likely correct and can be reconciled with the data in hand. As we shall now discuss, comparative genomics of *cif* genes, both at micro and macro-evolutionary scales, further support this conclusion [30,34].

CI evolution

Although *C. pipiens* provided the original CI model system, the simple pattern presented in Figure 1, which best illustrates the invasiveness of CI, is never seen in natural populations of this species. Wherever they come from, *C. pipiens* mosquitoes are always infected by *Wolbachia* (while uninfected specimens can occasionally be found in a cryptic species [40,41]). As a consequence, infected males never encounter uninfected females in nature and CI is only expressed in its most elaborate form: infected males and females can only produce offspring if they carry "compatible infections." Indeed, *C. pipiens* has long been known for harboring a large diversity of cross-incompatible mosquito lines [11], which are now known to carry closely related yet incompatible *Wolbachia* strains [42,43]. These are said to be "bidirectionally incompatible" if both directions of crosses result in embryo death. By

contrast, crosses between “unidirectionally incompatible” strains produce effective CI in only one direction, producing a pattern similar to that illustrated in Figure 1, although all individuals are infected.

How can this be? That different *Wolbachia* strains may harbor different compatibility types is easily explained in the framework of the TA model, especially in its lock-and-key formulation: incompatible *Wolbachia* strains will carry incompatible locks and keys. In this regard, *C. pipiens* is not unique: *Drosophila simulans*, among many other species, also carries several distinct *Wolbachia* strains, each with its own compatibility type [38]. In this fruit fly, compatibility relationships between lines can, at least in theory, be parsimoniously explained by variations at a single TA locus [44] (although some *D. simulans Wolbachia* genomes appear to include multiple cif paralogs [45]). In contrast, incompatibility patterns are so complex in *C. pipiens* that they cannot be explained with a single TA pair per *Wolbachia* genome [42,46]. Specifically, compatibility relationships are not all transitive in this system: two strains may be mutually compatible although they harbor distinct compatibility patterns with other strains [44]. Focusing on compatibility relationships among 19 wild-type *C. pipiens* lines [43], theoretical analyses grounded in the TA framework concluded that at least five TA pairs may co-occur in one *w*Pip genome [44]. Now that the CI genes have been identified, the time has come to test such theoretical predictions.

Although all infections from *C. pipiens* represent a monophyletic group of close *Wolbachia* relatives, fine-scale phylogenetic markers allow one to distinguish five clades within which crosses are most often compatible and between which they are most often incompatible [42,47]. Based on this phylogenetic and phenotypic diversity, Bonneau et al. [30] selected multiple mosquito lines collected worldwide to assess molecular variation of the *cif* genes and test their explanatory power with regard to compatibility patterns. Under the

hypothesis that these genes underlie CI diversity in *Culex*, they should be present in several distinct copies within each *Wolbachia* genome, and strains harboring different CI patterns should carry different *cif* repertoires. The *cid* data fully matched these predictions: in the *Culex* populations studied, *cidA* and *cidB* show tremendous variation in both sequence and copy number, resulting in large part from duplication and recombination events, possibly mediated by the prophage region where they occur. By contrast, the *cinA* and *cinB* genes were found to be monomorphic in the *w*Pip strains analyzed, indicating that incompatibilities in *Culex* are the result of *cid* but not *cin* variations.

While mutually incompatible strains should harbor different *cid* repertoires, as was indeed observed, the TA model does not predict that mutually compatible strains should carry exactly the same *cid* alleles. First, mutations may occur outside of the toxin / antidote interaction sites, which should not affect compatibility patterns and would thus be neutral as far as CI is concerned. Second, the TA model does not demand a strict one-to-one specificity of toxin-antidote interactions: some antidotes may inhibit more than one toxin. Both of these explanations may contribute to explain why a number of mutually compatible strains harbor different *cid* repertoires. Nevertheless, these strains happen to always share a common *cidA* variant that may represent a super-antidote, matching several distinct toxins [30]. Expressing these different Cid variants in an experimentally flexible *in vivo* system such as yeast, together with biochemical studies, should clarify this issue.

What does the *cid* polymorphism tell us about the evolutionary process of CI diversification? In other words, can *cid* molecular variations reveal how bidirectionally incompatible *Wolbachia* evolved? Past theoretical work has highlighted how much the genetic architecture of CI should affect this process [39,44,48,49]. The experimental confirmation that CI genes can occur in multiple copies [30] greatly simplifies the problem

from a theoretical standpoint: following duplication of a TA pair, redundancy between loci may allow new antidotes to emerge without compromising self-compatibility. This first step could be followed by the occurrence of matching mutations of the toxin, producing two distinct CI operons in a genome [44]. The possibility that some antidotes may inhibit more than one toxin opens yet other possible scenario, where either side may diversify first if the process goes through a broad spectrum phase before further restriction of specificity, as has been suggested in other TA systems [50].

The absence of polymorphism in the *cin* operon (the nuclease paralog of *cid*) rules out this locus as a driver of CI diversity in *Culex*. However, could this operon or other *cid* paralogs operate in other species? Comparative genomics among *Wolbachia* lineages indicate that some CI-inducing strains do not contain *cid* genes but only the *cin* paralogs [28,34]. Moreover, neither *cid* nor *cin*-related paralogs were found in a close relative of *w*Mel that does not induce CI [34,51], or in nematode *Wolbachia* strains that have become obligate mutualists [26,34]. These results support the involvement of both *cid* and *cin* operons in CI induction by *Wolbachia*, with evidence still emerging. Further, these two loci seem sufficient so far to explain all CI cases associated with *Wolbachia*.

This conclusion does not hold when symbiont lineages distant from *Wolbachia* are considered. Notably, *Cardinium* bacteria can induce CI but do not carry identifiable *cid* or *cin* genes, suggesting convergent evolution of CI [34,52]. However, a recent study suggested that TA-like systems showing a putative DUB activity can be found in a diverse array of endosymbionts, not only in *Cardinium* (albeit in a lineage where CI itself was not demonstrated), but also in other intracellular symbionts, such as *Rickettsia* and *Spiroplasma*, that are also known as manipulators of host reproduction [36]. Also notable is the presence of *cin* genes in non-CI but parthenogenesis-inducing *Wolbachia* strains [34]. Although these

results do not provide direct evidence that distant *cif* homologs are involved in CI or other forms of reproductive manipulation, they make this hypothesis worth exploring. Finally, the discovery of CI associated with a non-*Wolbachia* Alphaproteobacterium [53] provides another system where *cif* gene relatives should be sought.

Cui bono? Levels of selection and the origin of CI

From Cicero to the detective Columbo, asking "*Cui bono*?" (Who benefits?) has been a useful avenue of criminal and sociological investigation. This question is also relevant in a Darwinian framework and has prompted novel explanations of evolutionary oddities such as altruism and selfish genetic elements [54–56]. Applying this question to CI led early theorists to highlight the benefit it provides to *Wolbachia* itself, rather than its host, suggesting that the bacterium represents the right level of selection to understand how this phenomenon came to be [19]. Remarkably, the same early study was visionary in suggesting that the CI genes may be associated with mobile genetic elements: the discovery that they sit in a prophage supported this hypothesis [26–28,34,57]. In our view, this finding also makes it relevant to revisit the question of the adaptive significance of CI, and ask whether this phenomenon may have evolved in the first place for the benefit not of *Wolbachia*, but of the bacterium's own intragenomic parasites.

The hypothesis that TA systems, including restriction-modification systems or bacteriocins, constitute fundamentally selfish genetic elements has received ample support in the field of microbial evolution [58–63]. In brief, the idea is that TA systems make themselves addictive as soon as they enter a cell: the toxin molecule is typically more stable than the antidote, so that removing the source of both results in cell death. Although this property is not necessarily invasive (an element killing a host once it is lost increases its effective

transmission rate but will not, by itself, increase its frequency), it can promote invasion under certain circumstances, especially if the TA system is part of a horizontally transmitted mobile genetic element [64].

With regard to *Wolbachia*, it seems reasonable to assume that the nearly universal positioning of the CI genes within prophages [57,65] is not mere chance, but rather has some adaptive significance. So, who benefits from the CI genes, and more specifically, from their association with prophages? This particular location is not *a priori* adaptive at the *Wolbachia* level, but it may well be at the phage level: horizontally transmitted phages should more readily invade *Wolbachia* populations if they carry a TA system. The occurrence of distant *cif* relatives in several other bacterial lineages, where they sit in plasmids rather than prophages, argues against a purely viral origin of this gene family [36], and so does their relatedness to typical eukaryotic sequences [26,36,57]. However, the association of *cif* genes with phages in the *Wolbachia* lineage opens the possibility that they first invaded this clade as a phage adaptation, and only later became "CI genes."

As previously pointed out [66], the elimination of *Wolbachia* from maturing sperm establishes conditions for the evolution of such a selfish phage TA system toward genuine CI. If one simply assumes that the toxin is exported outside of the bacterium and can exert its deleterious effects on the host (eukaryotic) cell, then CI could easily arise. In particular, the paternal pronucleus, threatened by a stable toxin, would need fresh antidote from an infected egg to be able to participate to the first embryonic division. Such a situation would in turn make the bacterium invasive through CI, generating a convergence in the evolutionary interests of the phage and its bacterial host.

Concluding remarks and future perspectives

Recent research has provided a firm answer to the first of many critical questions regarding the molecular biology of CI (Outstanding Questions Box). There is indeed a general agreement that the principal *Wolbachia* CI genes have been found [26–29], and our present analysis concurs with past predictions that they encode toxins and antidotes interacting in a lock-and-key fashion [25]. Nevertheless, significant revisions of the basic TA model are needed to account for all observations. First, we suggest that CifB toxins may exert their deleterious effects not only in incompatible crosses during the first embryonic mitosis, but also in maturing sperm and other cell types, in which the presence of CifA antidotes would also be required. This hypothesis would explain why dual transgenic expression of the two genes in uninfected *Drosophila* males is required for them to survive (in the case of cid^{wPip}), or for CI to be transgenically expressed (in the case of cid^{wMel}) [27,28]. Patterns of molecular variation of the *cidA* and *cidB* genes [30] also suggest that some antidotes may match several toxin products, a conjecture that could be tested by direct assessments of CidA-B interactions in simplified *in vivo* or *in vitro* systems. Furthermore, these genetic data confirm the earlier speculation that single *Wolbachia* strains may carry multiple active CI operons, not only in *Culex* [67] but also in *Drosophila* [68].

While the genetics of CI have been clarified, its molecular mechanisms remain to be worked out (Box 2). Furthermore, the exact nature of the interaction between toxin and antidote proteins remains to be determined, although there are some indications of which residues might be involved [30]. Clarification of these issues will be needed to design artificial CI systems that may be used in biological control. Comparative genomics will provide a valuable complement to experiments to understand how CI actually works, and to investigate how it evolves. At a microevolutionary scale, further exploration of the *Culex* system will

clarify how different *Wolbachia* strains become mutually incompatible. On a macroevolutionary scale, genomic comparisons may further reveal which *cif* genes can actually induce CI, and what are the commonalities and specificities between these systems.

Building on the observation that the CI genes lie in a mobile genetic element (the WO prophage), we suggested they might have originally been selected as a phage invasive strategy and were later domesticated by the bacterium. In line with this hypothesis, distant homologues of the *cid* genes are present in other bacterial symbionts, and nearly always in association with phages or plasmids [36]. Pushing the reasoning one step further, one may envisage that the TA operon was initially costly for the phage, and only later became domesticated as an effective invasive strategy. CI would then be a case of a parasitic operon within a parasitic phage within a parasitic symbiont, each relying today on its past inner enemy. The observation that some insects, and many filarial nematodes, cannot live without *Wolbachia* [69–71] reinforces the idea that such "evolution through addiction" constitutes a never-ending process, producing the Russian dolls that all organisms seem to be.

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Boxes

Box 1: *Wolbachia* and Cytoplasmic Incompatibility

Perhaps the most crucial aspect of *Wolbachia* biology is the fact it is transmitted from mothers to offspring through the egg cytoplasm [72] although horizontal transfer may also occur [7,45]. Vertical transmission through the female germline will select *Wolbachia* traits that increase the fitness of infected females, or more technically, the number or the fitness of their infected daughters. CI can be interpreted within such a framework: by protecting infected embryos from the lethal effect of infected males' sperm, *Wolbachia* increases the relative brood size of infected females. Infected males pay a heavy price for CI (mating with uninfected females drastically reduces their own fertility) but this is costless for *Wolbachia* because males do not transmit the symbiont to future generations. Notably, only few uninfected females are sterilized through CI when *Wolbachia* is rare in the host population, so that a low frequency infection has low chances of invasion, unless it combines CI with other traits, such as protection of the host against pathogens [73]. Such protective effects are actually observed [74] and can also block the passage of human pathogens through insect vectors [75]. The ongoing "World Mosquito Program" makes use of this property: the massive release of CI-inducing mosquitoes allows the spread of the infection, which should reduce overall viral transmission rates [76] although the implementation and evolutionary outcome of this approach remain uncertain [77,78].

Box 2: CI molecular mechanisms

Whilst the CI effectors have been identified, the question of how they induce embryo death or rescue remains largely unanswered, and can be divided in two: how do *cif* toxins impede paternal chromosomes, and how do *cif* antidotes impede the toxins? Functional properties of the *cifB* genes are obviously relevant to the first question, as are characterizations of the CI phenotype at the cytological level. The earliest detected abnormality in CI crosses is improper deposition of maternal H3.3 histones on the paternal genome after protamine removal [79]. This deposition defect could be responsible for improper paternal chromosome condensation in prophase [22,80–82]. How could these features be related to *cif* genes activities? While bioinformatics predict a number of potential enzymatic properties for the various *cif* paralogs, the DUB domain of CidB stands at the moment as the strongest CI effector candidate: active-site mutations in CidB eliminate CI in transgenic insects as well as toxicity in yeast models [27]. Furthermore, different CidB repertoires induce different levels of CI defects at the cellular level [82]. Although the DUB activity may affect upstream components of the CI causal chain, interference with the ubiquitylation status of key chromatin or cell cycle regulators appears as an obvious hypothesis to link CidB to CI cytology.

However, the observation that some *Wolbachia* strains may lack *cid* genes but still induce CI suggests the DUB function is just one tool in a larger *cif* arsenal [26,28,34,36]. CinB appears as the most likely source of CI in this case, since active-site mutations block CinB toxicity in yeast [27]. But why and how would different enzymes generate the same CI phenotype? One hypothesis would be that the DUB and nuclease activities are two upstream components of a common causal chain. Simply put, cutting DNA and cleaving ubiquitin may constitute alternative ways to disturb paternal chromosome condensation. Definitive

demonstrations that CinB can induce CI, as well as identification of the CidB and CinB targets will be crucial to resolving these issues.

The fact that CI may be mediated by two distinct effectors, involving DUB or nuclease activities, is also relevant to investigate how CI antitoxins may function. CidA and CinA may inhibit DUB and nuclear activities, respectively, either through distinct pathways or alternatively, through a single mechanism, such as protein sequestration or relocalization. The latter hypothesis may explain why CidA can inhibit the toxicity of CidB without specifically reducing its DUB activity against model substrates [27]. Further characterization of the Cid and Cin protein structures and identification of the residues involved in the interaction between cognate partners of both operons appear as promising avenues of research to better understand how infected embryos get rescued from CI.

Figure captions

Figure 1. Cytoplasmic incompatibility in its simplest form: infected females are compatible with both infected and uninfected males, whereas uninfected females produce viable offspring only if they mate with uninfected males.

Figure 2. A schematic view of the toxin-antidote model. In immature sperm, *Wolbachia* (in pink) produces both a toxin (yellow particles) and its specific antidote (green particles). As *Wolbachia* is removed from maturing sperm into waste bags (w.b), the antidote, presumably unstable, is lost faster than the toxin. Upon fertilization of an uninfected egg (left part), the toxin is thus present and active, impeding the paternal chromosomes through direct or indirect interactions with chromatin or DNA, which results in embryo death. In infected eggs, antidotes of maternal origin bind the toxin and thus maintain embryo viability. Alternative CI mechanisms have been envisaged [21,22,83,84] but the model depicted here best accounts for all CI features [25], including its recently discovered genetic architecture [26–29].

Figure 3. A nomenclature proposal and schematic view of the putative CI operons structures. In this naming system, the *cif* (and Cif) terms designate CI genes (and proteins) in general, while genes from specific operon categories are called according to the enzymatic activity of the putative toxin (DUB, nuclease or both). The first and second gene within each operon are denoted A and B, respectively, and the *Wolbachia* strain is indicated as a superscript when relevant. The structure of several CI operons is shown to illustrate this system, with active-site residues labeled.

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Figure 1

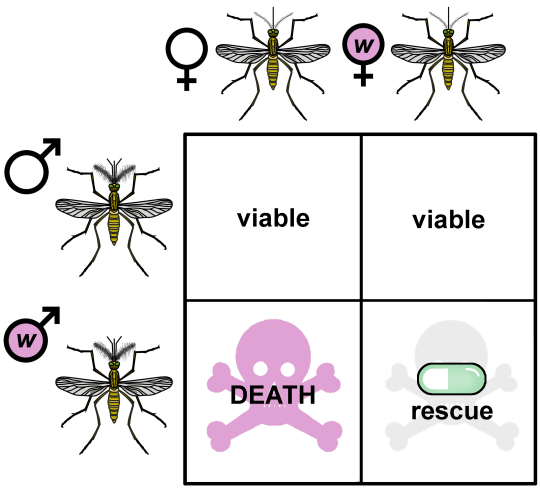


Figure 2

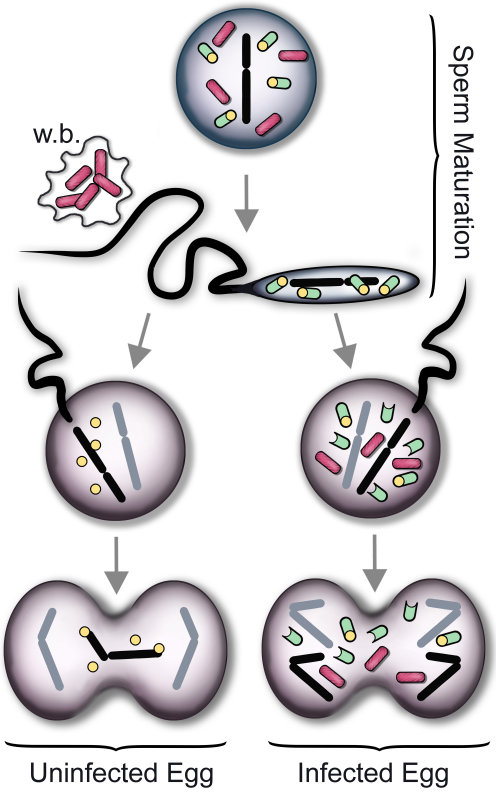
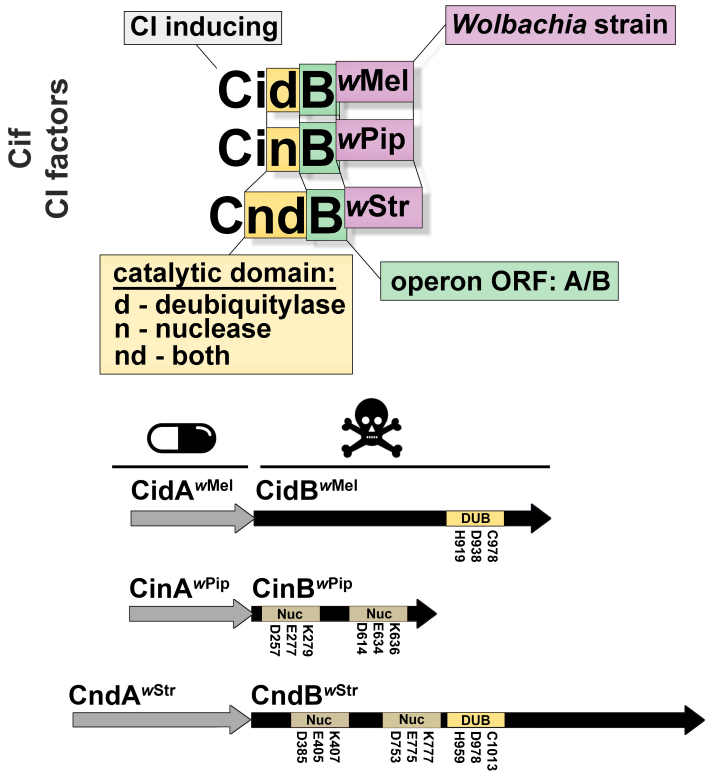


Figure 3



Highlights

Wolbachia are maternally inherited intracellular bacteria of many Arthropod species. They can invade populations through various strategies, including Cytoplasmic Incompatibility (CI), whereby the symbionts protect eggs from the lethal effect of infected males' sperm.

It has long been proposed that this phenomenon may rely on a toxin deposited by the bacterium before its elimination from maturing sperm, and an antidote, provided in an infected egg by the maternal symbiont.

Recent research toward the molecular basis of CI have turned the spotlight on two syntenic loci in a prophage region which recapitulate the CI phenotype and are organised in a typical toxin-antidote fashion.

This genetic architecture, archetypal of toxin-antidote systems promoting the spread of selfish mobile elements in free living bacteria, provides clues to the possible evolutionary origin of CI.

Outstanding questions

- What are the genes behind CI? Do they encode a toxin-antidote system?
- Among the various paralogs of the putative CI genes (*cif*, for CI factors) are only *cid* operons (including a DUB toxin) involved in CI, or also those including a nuclease toxin (*cin*) or toxins combining the two functions (*cnd*)?
- Is CI induced by CifB proteins alone? Why then is CI not recapitulated by sole transgenic expression of the main putative toxin gene (*cidB*) in *Drosophila*? Could this be explained by toxic effects of CidB outside of the first embryonic division, making the presence of its cognate antidote (CidA) required for fly survival or CI expression?
- How would CifB proteins induce embryo death? What targets are affected by the DUB activity of CidB? Are these in direct interaction with paternal chromosomes, or affecting upstream regulators of the cell-cycle? Do CidB and CinB proteins affect the same pathway?
- How do CifA proteins inhibit CifB toxicity without necessarily affecting their enzymatic activity? Could CifB sequestration or relocalization be involved?
- Can variation in *cid* genes, both in copy number and sequence variation, explain on their own the complex CI patterns in natural populations of the mosquito *C. pipiens*?
- How do mutually incompatible *Wolbachia* strains evolve? What is the contribution of gene duplication to this process? How specific is the *cifA* / *cifB* interaction, and does this vary across *cif* loci?
- Are all CI cases involving *cif* operons? What are the functions of their numerous distant homologues in other intra-cellular bacteria?
- How did CI evolve? Why are *cif* genes located in a prophage? Could CI derive from a selfish phage invading system?